Frequency estimation of disease-causing mutations in the Belgian population of some dog breeds - Part 1: shepherds

Frequentieschatting van ziekteveroorzakende mutaties in de Belgische populatie van enkele hondenrassen - Deel 1: herders

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ABSTRACT

In light of improving breeding advice, the frequency was estimated for all the disease-causing mutations that were known at the start of the study and that are potentially relevant for a group of dog breeds, which are relatively popular or in which the genetic diversity in Belgium is low to moderately low. In this study, the results for the German shepherd dog, Malinois, Lakenois, Groenendael, Tervuren, Australian shepherd and Border collie are presented. Disorders with a frequency high enough to warrant routine genotyping for breeding programs are (1) multidrug resistance 1 and hereditary cataract for the Australian shepherd, (2) degenerative myelopathy for the German shepherd dog, Malinois and Groenendael and (3) collie eye anomaly for the Border collie. In addition, the hyperuricosuria mutation described in the German shepherd dog was not found in its Belgian population, but was, to the authors' knowledge discovered for the first time in the Malinois.

SAMENVATTING

Om gerichter fokadvies te kunnen geven, werd de frequentie geschat van alle ziekteveroorzakende mutaties die bekend waren bij de start van de studie en potentieel relevant zijn voor een aantal hondenrassen die relatief populair zijn of waarvan de genetische diversiteit in België laag tot middelmatig laag is. De resultaten voor de Duitse herder, Mechelse herder, Lakense herder, groenendaeler, Tervurense herder, Australische herder en bordercollie worden hier besproken. Aandoeningen met een frequentie hoog genoeg om deze routinematig te genotyperen voor fokprogramma's zijn (1) "multidrug resistance 1" en "hereditary cataract" voor de Australische herder, (2) degeneratieve myelopathie voor de Duitse herder, de Mechelse herder en de groenendaeler en (3) "collie eye anomaly" voor de bordercollie. De hyperuricosurie-mutatie, beschreven bij de Duitse herder, werd in deze studie niet aangetroffen bij dit ras, maar werd volgens de auteurs wel voor het eerst vastgesteld bij de Mechelse herder.

INTRODUCTION

Common breeding practices in domestic dogs, combined with a small genetic basis in some breeds (Akita inu, Rough collie, Bichon frise, Laekenois, Papillon) have put the genetic diversity of some breeds under pressure. Examples of such practices are selection purely based on breed standards and the (over) use of a popular sire (matador breeding) (Calboli et al., 2008). Without any doubt, this has an impact on the frequency of disease-causing mutations in these breeds. Some breeds, such as the Berger des Pyrénées and the Braque Saint-Germain, have such a small and/or decreasing population size that they are considered as endangered (Leroy et al., 2006). In order to improve the genetic health of the breeds involved and to avoid the creation of clinically affected individuals as much as possible, breeding programs should be drafted in such a way that the genetic load imposed by hereditary disorders is reduced without reducing the genetic diversity even further. Ideally, these programs could even enhance the genetic diversity at the same time. In order to do so, a good knowledge of the typical disorders in a particular breed and of the frequency of the disease-causing variants is necessary (McGreevy and Nicholas, 1999).

Clinical (phenotypical) typing can only be used to estimate prevalences of genetic disorders, not fre-

quencies of causal mutations (Flint and Woolliams, 2008). It may result in an underestimation of frequencies due to (1) the fact that asymptomatic carriers of recessive disorders will not be identified and may silently accumulate in populations to a considerable degree before popping up in clinical cases, (2) the fact that some hereditary disorders are late-onset diseases (Awano et al., 2009; Zangerl et al., 2006), so that symptoms often show up after phenotyping and after the animal has already produced offspring (Flint and Woolliams, 2008), (3) a sometimes broad range in severity of the symptoms and (4) possible confusion with other diseases. Since the goal of this study was to estimate the frequency of causal mutations and give breeding advice accordingly, genotyping by using a DNA test was the chosen tool.

The most important limitations of the genotyping technique are that the causative mutation(s) of a disorder is not yet known or that a closely linked marker that can be reliably used in marker assisted selection, is absent. This is especially true for polygenic/multifactorial disorders, several of which are sadly enough common in a wide range of dog breeds (Online Mendelian Inheritance in Animal (OMIA)). As a consequence, nearly all DNA tests available to date are for monogenic traits. An example of a polygenic disorder, for which there is a DNA test, is the one for HD, which only explains a small percentage of the variation (Friedenberg et al., 2011). However, it should be noted that some disorders can be caused independently by several mutations and that a DNA test may only detect one of them (Mellersh et al., 2009). This genetic heterogeneity of a disorder can be either caused by different mutations in the same gene (allelic heterogeneity) or by mutations in different genes (locus heterogeneity) (Gelehrter et al., 1998). This means that an animal may be genotyped homozygous wildtype (Wt/Wt) in a certain DNA test and still develop the disorder due to another (unknown) mutation.

Due to the lack of specific mutant allele frequencies of the population at hand, extrapolations from studies in other populations are frequent. Although useful, it may occasionally lead to serious deviation from the actual situation. Allele frequencies are subject to time- and space-related fluctuations caused by several factors, of which the popular sire effect is an important one in dog breeding (Mellersh, 2008; Leroy, 2011). Information from related breeds is sometimes used out of necessity or convenience. This practice is even more prone to error due to a larger genetic divergence and due to the fact that the relationship between breeds is not always clear. Extrapolation may however be an asset in case of data-poor breeds. For example, in this study, DNA tests developed for the popular German shepherd dog were used on Malinois, Laekenois, Groenendael and Tervuren, often collectively called Belgian shepherds. With the exception of the Malinois, the populations of these local breeds are small, and only very few genetic studies of these breeds have been reported.

In the present study, DNA tests were performed in 17 dog breeds. In this first part, the results obtained for seven shepherd breeds (Australian shepherd, German shepherd dog, Groenendael, Laekenois, Malinois, Tervuren and Border collie) are discussed. The results of another ten dog breeds will be discussed in part two.

MATERIALS AND METHODS

Breed selection

The breeds included in this study were part of a larger group of breeds, of which the genetic diversity was studied in the framework of an action program of the Flemish Government (Wijnrocx et al., 2012). In this program, 23 breeds were studied. Of these, the more popular breeds and breeds with the lowest genetic diversity, for which at least two DNA tests were available at the start of the study, were chosen for inclusion in this frequency study.

Samples and DNA extraction

The blood samples used, originated from a pool of samples (stored at -20 °C) delivered by veterinarians, animal clinics, breeders and breeder associations from all over Belgium for routine genotyping. In order to keep the bias at its lowest, closely related animals (relation >12.5%) were excluded. However, this was not always possible due to the lack of data or to small population size. The aim was to gather at least fifty samples (hundred alleles) for each breed. However, this number was not reached for the Australian shepherd (n = 32) and the Laekenois (n = 27).

For DNA-extraction, whole blood samples collected in EDTA-tubes, were used. One hundred µl of each sample were washed with 500 µl Tris-HCl-EDTA until a clean pellet of white blood cells remained. The cells were resuspended in a lysis buffer containing proteinase K and DNA was released during a forty-five-minute incubation step at 56 °C. The enzyme was afterwards inactivated during a ten-minute incubation step at 95 °C.

Disorders and DNA tests

The disorders were selected based on the data found in the literature for each breed. In total, 14 different tests were performed and the mutant and wild-type allele frequencies were calculated for each disorder. Based on these results, it was checked whether the populations of the present study deviated from the Hardy-Weinberg equilibrium.

All assays were first validated by sequencing (golden standard) and later performed with easier, quicker and/or cheaper techniques such as PCR (-RFLP) followed by gel electrophoresis or qPCR

Table 1. Overview of the performed DNA tests. Ta indicates the annealing temperature and F and R indicate forward and reverse primers respectively. TR indicates Texas Red.

Disorder: Colly eye anomaly (CEA)			**OMIA ID:** 000218-9615
Gene symbol: NHEJ1	**Inheritance:** Autosomal recessive	**dbvar ID:** nsv1397887	**Reference:** Parker et al., 2007
Assay: PCR with F/R_1/R_2 primers and gel electrophoresis		**Ta:** 64°C	**Amplicon:** Wt: 322 bp; Mt: 200 bp
Primers: F: 5'-tttggttgcccagtcgatca-3'; R1: 5'-cccctgtttgccgtttaatagatgt-3'; R2: 5'-ccaatcatccagcccagcat-3'			
Disorder: Cyclic neutropenia (CN)			**OMIA ID:** 000248-9615
Gene symbol: AP3B1	**Inheritance:** Autosomal recessive	**dbSNP ID:** ss1961068737	**Reference:** Benson et al., 2003
Assay: PCR with F/R primers and sequence analysis with F primer		**Ta:** 58°C	**Amplicon:** 266 bp
Primers: F: 5'-tttcttggaaggctaagtgg-3'; R: 5'-gaaggtccatgctctctcttac-3'			
Disorder: Degenerative myelopathy (DM)			**OMIA ID:** 000263-9615
Gene symbol: SOD1	**Inheritance:** Autosomal recessive	**dbSNP ID:** ss1961068758	**Reference:** Awano et al., 2009
Assay: qPCR with dual labeled probes		**Ta:** 56°C	**Amplicon:** 192 bp
Primers: F: 5'-cttccactttcttgtgattg -3'; R: 5'-caccttgtgtattatctccaa-3'		**Probes:** Wt: HEX-cgccttcagtcagcc-BHQ1; Mt: TR-cgcctttagtcagccc-BHQ2	
Disorder: Hereditary cataract (HC)			**OMIA ID:** 001758-9615
Gene symbol: HSF4	**Inheritance:** Autosomal dominant	**dbSNP ID:** ss1961068742	**Reference:** Mellersh et al., 2006
Assay: PCR with F/R_1 primers and sequence analysis with R_2 primer		**Ta:** 58°C	**Amplicon:** 218 bp
Primers: F: 5'-tggccccaaacgagtgtgat-3'; R_1: 5'-ttagtggtggtctatctgcaaaagtgg-3'; R_2: 5'-aggctgttgggcattcctg-3'			
Disorder: Hyperuricosuria (HUU)			**OMIA ID:** 001033-9615
Gene symbol: SLC2A9	**Inheritance:** Autosomal recessive	**dbSNP ID:** ss1961068738	**Reference:** Bannasch et al., 2008
Assay: qPCR with dual labeled probes		**Ta:** 63°C	**Amplicon:** 101 bp
Primers: F: 5'-ccaaggagatccgtggc-3'; R_1: 5'-cttccccagcagctcag-3'		**Probes:** Wt: FAM-ccatcttcatctgcatcggtgt-BHQ1; Mt: TR-catcttcatcttcatcggtgtgttc-BHQ2	
Disorder: Imerslund-Gräsbeck syndrome (IGS-AS)			**OMIA ID:** 000565-9615
Gene symbol: AMN	**Inheritance:** Autosomal recessive	**dbSNP ID:** ss1961068745	**Reference:** He et al., 2005
Assay: PCR with F/R primers and sequence analysis with R primer		**Ta:** 64°C	**Amplicon:** 376 bp
Primers: F: 5'-ggcttggaaggaaggcccccca-3'; R: 5'-caaggcggggagcctccgaa-3'			
Disorder: Imerslund-Gräsbeck syndrome (IGS-B)			**OMIA ID:** 001786-9615
Gene symbol: CUBN	**Inheritance:** Autosomal recessive	**dbSNP ID:** ss1961068736	**Reference:** Owczarek-Lipska et al., 2013
Assay: PCR with F/R primers and sequence analysis with F-Primer		**Ta:** 62°C	**Amplicon:** 248 bp
Primers: F: 5'-gacttcaaaccccaggacaatc-3'; R: 5'-acatgaatggtgagggtaaacg-3'			
Disorder: Multidrug resistance 1 (MDR1)			**OMIA ID:** 001402-9615
Gene symbol: ABCB1	**Inheritance:** Autosomal partially dominant	**dbSNP ID:** ss1961068749	**Reference:** Mealey et al., 2001
Assay: qPCR with dual labeled probes		**Ta:** 56°C	**Amplicon:** 180 bp
Primers: F: 5'- gataggttgtatatgttggtg -3'; R: 5'-ctaagatcagtgccacaa-3'		**Probes:** Wt: HEX-tgcaaagctatctgtcatgtttc-BHQ1; Mt: TR-ttgcaaagctgtcatgtttcc-BHQ2	
Disorder: Mucopolysaccharidosis VII (MPS VII)			**OMIA ID:** 000667-9615
Gene symbol: GUSB	**Inheritance:** Autosomal recessive	**dbSNP ID:** ss1961068744	**Reference:** Ray et al., 1998
Assay: qPCR with dual labeled probes		**Ta:** 62°C	**Amplicon:** 115 bp
Primers: F: 5'-cagcaagttggtccagagc-3'; R: 5'-agacgatggtccctggc-3'		**Probes:** Wt: FAM-tgtcctcctgccgtattaccct-BHQ1; Mt: TR-tgtcctcctgccatattaccct-BHQ2	
Disorder: Neuronal ceroid lipofuscinosis 5 (NCL5)			**OMIA ID:** 001482-9615
Gene symbol: CLN5	**Inheritance:** Autosomal recessive	**dbSNP ID:** ss1961068753	**Reference:** Melville et al., 2005
Assay: PCR with F/R primers and sequence analysis with R primer		**Ta:** 60°C	**Amplicon:** 214 bp
Primers: F: 5'-aaagcgggacaatgaaacag-3'; R: 5'-tcccaagtaggtaggttctcca-3'			
Disorder: Neuronal ceroid lipofuscinosis 6 (NCL6)			**OMIA ID:** 001443-9615
Gene symbol: CLN6	**Inheritance:** Autosomal recessive	**dbSNP ID:** ss1961068755	**Reference:** Katz et al., 2011
Assay: PCR with F/R primers and sequence analysis with F primer		**Ta:** 64°C	**Amplicon:** 189 bp
Primers: F: 5'-tgcaccagaagcggaagc-3'; R: 5'-tgctgacgtggagggtgtaga-3'			
Disorder: Primary Lens Luxation (PLL)			**OMIA ID:** 000588-9615
Gene symbol: ADAMTS17	**Inheritance:** Ausotomal recessive	**dbSNP ID:** ss1961068740	**Reference:** Farias et al., 2010
Assay: PCR with F/R primers and sequence analysis with F primer		**Ta:** 58°C	**Amplicon:** 169 bp
Primers: F: 5'-gtctccctcacaagcta-3' ; R: 5'-ggcatatggatagctgtatc-3'			
Disorder: Renal cystadenocarnicoma and nodular dermatofibrosis (RCND)			**OMIA ID:** 001335-9615
Gene symbol: FLCN	**Inheritance:** Autosomal dominant	**dbSNP ID:** ss1961068743	**Reference:** Lingaas et al., 2003
Assay: qPCR with dual labeled probes		**Ta:** 60°C	**Amplicon:** 111 bp
Primers: F: 5'-ctgacctccttgacaag-3'; R: 5'-ccagtataccatgtctactc-3'		**Probes:** Wt: FAM-catgccttcatacctccttt-BHQ1; Mt: TR-atgccttcgtacctcctt-BHQ2	
Disorder: X-Linked hypohidrotic ectodermal dysplasia (XHED)			**OMIA ID:** 000543-9615
Gene symbol: EDA	**Inheritance:** X-Linked recessive	**dbSNP ID:** ss1961068763	**Reference:** Casal et al., 2005
Assay: PCR-RFLP with ScrFI		**Ta:** 64°C	**Amplicon:** 194 bp
Primers: F: 5'-tcccttcttgttgcctctcacc-3'; R: 5'-ccatcttcaccgcaatcttctg-3'		**Fragment Lengths:** Wt: 146/25/23 bp; Mt: 169/25 bp	

with dual labeled probes when applicable. All specifics, as well as the performed (alternative) test for each disorder can be found in Table 1.

PCR-sequencing

All assays were first validated through PCR followed by sequencing. For the PCR, a mix containing 2.0 µl of the DNA, 5.7 µl sterile, enzyme-free water (MQ; Thermo Fisher Scientific Inc., Erembodegem, Belgium), 1.0 µl 10x FastStart buffer (Roche Diagnostics, Vilvoorde, Belgium), 1.0 µl primers (5 µM each, Integrated DNA Technologies, Leuven), 0.2 µl deoxynucleoside triphosphate mixture (dNTP's; 10 mM each, Gentaur, Kampenhout, Belgium) and 0.1 µl FastStart polymerase (5 U/µl, Roche Diagnostics, Vilvoorde, Belgium) was made. Hereafter, the remaining 8.0 µl PCR product was cleaned up with 0.6 µl Exo-AP solution (10 µl 20 U/µl exonuclease I and 20 µl 5 U/µl antarctic phosphatase in a 30 µl stock; Bioké, Leiden, the Netherlands), digesting the remaining primers and nucleotides (30' at 37°C, 15' at 80°C, hold at 4°C). This product was used as template for the sequencing reaction. To make the sequence mix, 5x sequence buffer (Life Technologies, Ghent, Belgium), ReadyReaction mix (RR-mix, Life Technologies, Ghent, Belgium), primer (2 µM, Integrated DNA Technologies, Leuven, Belgium), MQ and GC-solution (Roche Diagnostics, Vilvoorde, Belgium) was added to the template product. After this, the mix was sent to Eurofins Scientific (Germany) for running the electrophoresis.

PCR-gel electrophoresis

The detection of INDELs (insertions and/or deletions) longer than 20 bp was performed by PCR followed by gel electrophoresis (PCR-GE). The same PCR mix was used as described for PCR-sequencing. After the PCR, the mix was loaded on a 2%-agarose gel. The length of the fragments is viewed in Table 1.

PCR-RFLP

For mutations creating or abolishing a restriction enzyme recognition site, a PCR- restriction fragment length polymorphism (PCR-RFLP) assay was performed. The PCR reaction was performed as described above, followed by a restriction digest with the respective enzyme cutting one of both Wt/Mt alleles. This was done overnight with 5 U of enzyme according to the instructions of Bioké (Leiden, the Netherlands). The end product was loaded on a 3% agarose gel to separate the different fragment lengths (Table 1).

qPCR with dual labeled probes

The standard mix for the real-time PCR consists of 4.7 µl MQ, 1.0 µl 10x FastStart buffer, 1.0 µl primers (5 µM each), 0.5 µl of each probe (10 µM, Integrated DNA Technologies, Leuven, Belgium), 0.2 µl dNTP's (10 mM each), 0.1 µl FastStart Polymerase (5 U/µl) and 2.0 µl DNA. The mix was run through a PCR program with conditions specific to the disorder (Table 1), during which a curve was drafted by the Bio-Rad CFX96 C100 Touch™ Thermal Cycler real-time PCR.

RESULTS AND DISCUSSION

All genotyped disorders for each breed, as well as the mutations, for which routine genotyping is advised, are shown in Table 2. Details on causal mutations and genes can be found in Table 1. An overview of all disorders, for which at least one mutant allele was found, can be viewed in Table 3. No deviation in the Hardy-Weinberg equilibrium was found for any of the disorders in the genotyped breeds.

A personal advice is drafted concerning the use of DNA-tests. Routine genotyping in breeding dogs is not recommended when the mutation was not found in the present samples or when the mutant allele frequency is (very) low. However, it might be advisable for breeders to test for a condition anyway, e.g. when a precedent is known in the family or line or when an animal has been imported from another population (country), even if the mutant allele frequency is low. Genotyping may assist in making a deliberate partner choice, thus preventing the birth of affected animals and over time reducing the mutant allele frequency. By seeking individual genetic counseling, a (further) decrease in the genetic diversity can be prevented.

Australian shepherd

Six different tests were performed on the samples of the Australian shepherd. Imerslund-Gräsbeck syndrome in the Australian shepherd (IGS-AS), an autosomal recessive disorder causing cobalamine malabsorption, was previously described in a family of Australian shepherds in the USA (He et al., 2005), and neuronal ceroid lipofuscinosis 6 (NCL6), a neurodegenerative autosomal recessive disorder, was found in the USA (O'Brien and Katz, 2008) and the UK (Bond et al., 2013). The mutant allele was not found in the sample of the present study (n = 31 for IGS-AS and n = 32 for NCL6) (Table 2). Given the small sample size of this study, the results should be interpreted with care, although it can be concluded that the frequency of IGS-AS and NCL6 might be (very) low in the Belgian population. No frequency data is available for other populations.

Collie eye anomaly (CEA) is a mild autosomal recessive disorder of the eye, present in a variety of breeds. It is caused by a disruption in the development of the retina, chorion and sclera (Parker et al., 2007). Two carriers for CEA were detected for the Australian shepherd (n = 32) (Table 3), while a higher frequency

Table 2. Breeds and general outcome of the DNA tests. A "+" indicates at least one mutant allele was found in the breed. A "-" indicates the mutation was not found in the breed. Disorders that should be routinely tested are indicated with a "*".

Breed	Outcome	Tests
Australian shepherd	+	Hereditary cataract* (HC), multidrug resistance 1* (MDR1), collie eye anomaly (CEA), hyperuricosuria (HUU)
	-	Imerslund-Gräsbeck syndrome (IGS-AS), neuronal ceroid lipofuscinosis 6 (NCL6)
Border collie	+	CEA*, IGS-B, MDR1
	-	Cyclic neutropenia (CN), NCL5, primary lens luxation (PLL)
German shepherd dog	+	Degenerative myelopathy* (DM), mucopolysaccharidosis type VII (MPS VII)
	-	HUU, renal cystadenocarcinoma and nodular dermatofibrosis (RCND), X-linked anhidrotic ectodermal dysplasia (XHED)
Groenendael	+	DM*
	-	HUU, MPS VII, RCND, XHED
Laekenois	-	DM, HUU, MPS VII, RCND, XHED
Malinois	+	DM*, HUU
	-	MPS VII, RCND, XHED
Tervuren	-	DM, HUU, MPS VII, RCND, XHED

was estimated for the Border collie (see below). In Switzerland, in a clinical retrospective study, a very low prevalence of affected Australian shepherds (only one affected dog was found out of the 571 samples) was found, while a higher percentage of 0.7% was found in the Border collie (Walser-Reinhardt et al., 2009). The results of the present study are in line with these findings.

Another defect of the eye is hereditary cataract (HC), characterized by blurring of the lens. This is an early-onset cataract and probably has an autosomal dominant mode of inheritance (Mellersh et al., 2006). It should be noted that other forms of cataract are present in the Australian shepherd (Mellersh et al., 2009), and recently, a new locus associated with cataract in this breed has been found (Ricketts et al., 2015). In this study, a mutant allele frequency of 7.8% was calculated (n = 32) (Table 3). The fairly high frequency of an early-onset dominant disorder may be explained by the fact that it is a mild disease, and breeders do not specifically select against it. No frequency data is available in other countries. Given the fairly high frequency, it should be advised to test breeding dogs routinely.

Hyperuricosuria (HUU) or urolithiasis is an autosomal recessive disorder. In the Dalmatian, all individuals are homozygous mutant (Bannasch et al., 2008) and the causal mutation has also been found in ten other breeds, including the Australian shepherd (Karmi et al., 2010). In this study, two heterozygous individuals were found in the 32 tested Australian shepherds, leading to a mutant allele frequency of 3.1% (Table 3). A similar frequency of 3.5% has been estimated in the USA (Karmi et al., 2010).

Animals homozygous for the multidrug resistance 1 (MDR1) mutation show a high sensitivity to ivermectin and several other chemicals due to a lack of P-glycoproteins in the blood-brain barrier (Ta ble 1). The animals are quickly overdosed and show neurotoxic symptoms. The trait is partially dominant, in that heterozygous animals are more sensitive, but much less than the homozygous mutant ones (Mealey et al., 2001). In the present study, a very high mutant allele frequency (q = 29.7%) was found in the Australian shepherd, which stands in contrast to the 0.5% found in the Border collie (see below). Studies performed in populations in Germany (Geyer et al., 2005; Gramer et al., 2011), the USA (Neff et al., 2004; Mealey and Meurs, 2008) and the UK (Tappin et al., 2012), showed similar frequencies for the Australian shepherd between 17.0% and 46.0%. Routine testing for this mutation should be encouraged, in the first place to support the choice of medication for animals and secondly to gradually reduce the frequency of the mutant allele. The latter should be performed with precaution due to the high frequency, and only to the degree that it does not endanger the genetic diversity of the breed.

Border collie

Six previously described hereditary disorders were tested in the Border collie. Cyclic neutropenia (CN) is an autosomal recessive inheriting disorder (Benson et

al., 2003) and has been described in the collie, a breed closely related to the Border collie (Lund et al., 1967; Reynold et al., 1971; Dale et al., 1972). It is characterized by oscillations in the number of neutrophils and other blood cells (Benson et al., 2003). This disorder in the Border collie was tested in the present study, but no mutant allele was found in the studied population (n = 95). As of yet, no frequency data is available in other countries.

For neuronal ceroid lipofuscinosis 5 (NCL5), an autosomal recessive neurodegenerative disease (Melville et al., 2005), the mutation was not found in the Belgian population (n = 95). The frequency of the mutant allele was estimated to be 3.5% in Australia. The mutation is relatively wide spread in that country, because some of its champions that carried the mutation, had frequently been used for breeding purposes (Melville et al., 2005). Mizukami et al. (2011) found a frequency of 8.1% in Japan. This high frequency is probably due to the importation of a few Australian champions that carried the mutant allele (founder effect). Moreover, analysis has suggested that the mutation originated in Australia (Mizukami et al, 2012a). The lack of a mutation in the Belgian population can probably be explained by the fact that there has been no contact between the Belgian and the Australian/ Japanese population. In light of these results, it is highly recommended to genotype imported animals.

Primary lens luxation (PLL) is an autosomal recessive eye condition (Farias et al., 2010). The causal mutation was first found in the miniature bull terrier, Jack Russel terrier and Lancashire heeler and is additionally present in 14 other breeds (Gould et al., 2011). This mutation is not seen in some other breeds with PLL, suggesting this condition is genetically heterogeneous (Gould et al., 2011). Because this mutation is present in the Lancashire heeler, which is a closely related breed to the Border collie (Gould et al., 2011; Parker et al., 2007), this test was included in this study. However, the mutation was not found in the tested population (n = 95). Gould et al. (2011) genotyped one Border collie with PLL and found it Wt/Wt. These results indicate that PLL in the Border collie is caused by a different mutation than the one described.

Ninety-five Border collies were genotyped for CEA, and one (1.1%) homozygous mutant (Mt/Mt) and 15 (15.8%) heterozygous (Wt/Mt) animals and a mutant allele frequency of 8.9% were found (Table 3). A clinical study in Switzerland estimated 0.7% affected Border collies (Walser-Reinhardt et al., 2009), which is in line with the 1.1% homozygous mutant dogs of the present study. As it concerned a clinical study, heterozygotes could not be identified. In previous studies, a frequency of 2.0-3.0% in the UK (Bedford, 1982, referred in Lowe et al., 2003) and the USA (Lowe et al., 2003) has been reported. Given the relatively high frequency, genotyping in animals used for breeding purposes should be encouraged.

The causal mutation of IGS in the Border collie (IGS-B) (Owczarek-Lipska et al., 2013) is situated in a different gene than the one in the Australian shepherd (He et al., 2005). A carrier (Wt/Mt) frequency of 4.3% was estimated in the studied population (n = 94), which is in line with the 6.2% Owczarek-Lipska et al. (2013) found in Switzerland.

Multidrug resistance 1 (MDR1) in the Border collie is caused by the same mutation as in the Australian shepherd. A mutant allele frequency of 0.5% was found in the genotyped population (n = 95) (Table 3), which is in contrast with the 29.7% found for the Australian shepherd (see above). In the USA (Mealey and Meurs, 2008) and in Germany (Gramer et al., 2011) a mutant allele frequency of more or less 1.0% has been reported. In Japan, 0.5% heterozygotes and 0.3% homozygous mutants were found (Mizukami et al., 2012b).

German shepherd dog

The German shepherd dog was genotyped for five different disorders. No mutation was found in the sample for HUU (n = 100), renal cystadenocarcinoma and nodular dermatofibrosis (RCND) (n = 96) and X-linked anhidrotic ectodermal dysplasia (XHED) (n = 98) (Table 2). Hyperuricosuria is present in the German shepherd dog in the USA population (Karmi et al., 2010), though at a very low rate, i.e. one carrier and one affected dog out of 114 animals genotyped. Renal cystadenocarcinoma and nodular dermatofibrosis, a renal tumor inheriting in an autosomal dominant pattern (Lium and Moe, 1985; Moe and Lium, 1997), and XHED, an X-chromosome linked recessive trait (Casal et al., 1997; Casal et al., 2005) have been described previously in the German shepherd dog. So far, no frequency determination has been performed for the two last mentioned disorders.

Degenerative myelopathy (DM) is a fatal late-onset neurodegenerative disorder of the spinal cord with an incomplete penetrance and is found in many different breeds, including the German shepherd dog. Symptoms, typically starting with ataxia in the hind limbs, only manifest around the age of six to eight years (Awano et al., 2009). The results of the present study indicate a very high frequency (q = 21.4%) for DM in the Belgian population of the German shepherd dog (n = 98) (Table 3). The high frequency may be explained by the fact that it concerns a late-onset disease and in many cases, animals have already been used for breeding purposes before the symptoms appear. A mutant allele frequency of 15.0% was found in a population consisting of Belgian, German and Dutch dogs (Broeckx et al., 2013), as well as a 17.0% frequency in an Italian population (Capucchio et al., 2014). In a USA population, the frequency was determined at 37.0% (Zeng et al., 2014). The Orthopedic Foundation for Animals (OFA) reported 16.0% homozygous mutant dogs and 31.6% carriers. It should be noted though that these tested dogs were presented in an animal clinic, probably creating bias. Notwith-

Table 3. Overview of the tests with at least one mutant allele detected for each breed with the total number of dogs tested (Total), the number of homozygous normal (Wt/Wt), heterozygous (Wt/Mt) and affected (Mt/Mt) individuals and the mutant allele frequency (q).

Breed	Test	Total	Wt/Wt	Wt/Mt	Mt/Mt	q (%)
Australian shepherd	CEA	32	30	2	0	3.1
	HC	32	27	5	0	7.8
	HUU	32	30	2	0	3.1
	MDR1	32	15	15	2	29.7
Border collie	CEA	95	79	15	1	8.9
	IGS-B	94	90	4	0	2.1
	MDR1	95	94	1	0	0.5
German shepherd dog	DM	98	62	30	6	21.4
	MPS VII	96	95	1	0	0.5
Groenendael	DM	51	49	2	0	2.0
Malinois	DM	73	63	9	1	7.5
	HUU	74	70	3	1	3.4

standing, it does indicate a similar high frequency for DM in the German shepherd dog in the USA as seen in Europe. It is strongly recommended to routinely genotype for this condition in dogs used for breeding purposes.

Mucopolysaccharidosis type VII (MPS VII) is an autosomal recessive lysosomal storage disease with a progressive course, ending in mortality (Ray et al., 1998). It was first described in a mixed-breed German shepherd dog (Haskins et al., 1984). Only one carrier of the *GUSB* mutation was present in the 96 samples of the present study (Table 3). Hence, it can be concluded that the mutation is present at a low to very low frequency in the Belgian population of the German shepherd dog. This conclusion can probably be extended to the general population of German shepherd dogs, as up till now, only two cases (one in a mixed-breed and one in a German shepherd) of MPS VII have been reported (Haskins et al., 1984; Silverstein et al., 2004). Broeckx et al. (2013) did not find the mutant allele.

Belgian shepherds

The Groenendael (n = 51), Laekenois (n = 27), Malinois (n = 74) and Tervuren (n = 56) are often collectively called Belgian shepherds. For these four breeds, in this study, DNA tests developed for the more popular German shepherd dog were used, since they fall under the same breed group by the Fédération Cynologique Internationale (FCI). Little data is available on the Belgian shepherds and no DNA tests are available in the public domain that apply specifically to them. No mutant allele was found in any of these breeds for MPS VII, RCND and XHED (Table 2). A few clinical cases with symptoms resembling and strongly indicating XHED have been described in the Belgian shepherd (Muller and Kirk, 1976), with no further specification of the breed.

Two DM carriers were identified in the Groenendael population of the present study and a mutant allele frequency of 7.5% was estimated for the Malinois (Table 3). The OFA reported the presence of DM in three Belgian shepherd breeds, with a frequency of 3.6% for the Malinois (n = 28), 37.5% for the Groenendael (n = 8) and 9.1% for the Tervuren (n = 11). Unlike the findings by the OFA, the mutation was not present in the Tervuren population of the present study. It should be noted that the used samples of the OFA originate from animal clinics only, probably leading to an overestimation of frequency. However, it can be concluded that the DM mutation is present in all three breeds of the USA population. Recently, a mutant allele frequency of 6.0% has been calculated for a USA population of 101 Malinois (Zeng et al., 2014). This is a result similar to what was found in the Belgian population of the present study. Because of the fairly high frequency in the Malinois, which is however not quite as high as the one found in the German shepherd dog, and because of the severity of the disorder, it is strongly recommended to routinely test for this disorder in the Malinois and the Groenendael.

Next to the Dalmatian and the Australian shepherd, HUU is present in the German shepherd dog (Karmi et al., 2010). Therefore, the Belgian shepherds were also genotyped for this trait. Three carriers and one affected dog were found in the Malinois samples (n = 74) (Table 3). The mutation was solely found in the Malinois and was absent in the other three shepherd breeds.

GENERAL DISCUSSION AND CONCLUSION

For XHED, an X-chromosome linked disorder, a mutant allele was neither found in the Belgian nor in the German shepherd dog (see also part 2).

A low mutant allele frequency may be explained by the fact that males only carry one X-chromosome. Provided that the disease is early-onset and has a 100% penetrance, carrying a mutant allele will be phenotypically identifiable and the dog will most likely be excluded from breeding programs. Since males sire more offspring than females, the exclusion of affected male dogs will have a great impact on the prevalence of these disorders, resulting in a lower frequency.

A similar conclusion can be reached for the autosomal dominant trait RCND (in the Belgian shepherds and the German shepherd), which is an early-onset disorder with a full penetrance of clear symptoms. The mutant allele cannot unknowingly be passed on from generation to generation. No mutant allele was found for this disorder. HC probably has an autosomal dominant mode of inheritance and is an early-onset disease, though a mutant allele frequency of 7.8% was found. This may be explained by the fact that the disorder is relatively mild; hence, there is no or only weak selection against it. A very high frequency of 29.7% for the MDR1 mutation was found in the Australian shepherd. While being a partially dominant trait, MDR1 is not a disease per se and only becomes clear when the affected dog is given the wrong medication.

For most of the genotyped dogs, no or only one disease-causing mutation was found. However, several dogs carried two or even three of the studied mutations. One Australian shepherd tested heterozygous for HC, MDR1 and CEA. The frequencies found in this study were generally in line with those described for populations elsewhere, but there is one that shows a significant deviation, thus proving population specific dynamics and the importance of this type of study. For DM in the Groenendael (q = 3.9%) and the Tervuren (q = 0.0%), a (very) low frequency was found. This is in contrast with the high frequencies found by the OFA of 37.5% and 9.1% for the Groenendael and the Tervuren, respectively. This is a noticeable difference, even considering the selection bias by the OFA.

Neuronal ceroid lipofuscinosis 5 in the Border collie is a clear example of the founder and popular sire effect (Mizukami et al., 2012a). It might also explain why the mutation was not seen in the Belgian population of the present study.

High enough frequencies to warrant routine genotyping in breeding programs were found for DM in the German shepherd dog, the Malinois and the Groenendael, MDR1 and HC in the Australian shepherd and CEA in the Border collie. Genotyping can be used to make a deliberate partner choice or sometimes to exclude animals from breeding programs. Heterozygotes, and in case of a very high frequency also homozygote mutants, should not be routinely excluded from breeding programs, since this may (further) endanger the genetic diversity. They may be paired with homozygous normal individuals, thus slowly but surely decreasing the mutant allele frequency. Individual genetic counseling can be sought after getting the genotyping results.

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